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# Specific patterns of changes in wheat gene expression after treatment with three antifungal compounds

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**Abstract**

The two fungicides azoxystrobin and fenpropimorph are used against powdery mildew and rust diseases in wheat (*Triticum aestivum* L). Azoxystrobin, a strobilurin, inhibits fungal mitochondrial respiration and fenpropimorph, a morpholin, represses biosynthesis of ergosterol, the major sterol of fungal membranes. Although the fungitoxic activity of these compounds is well understood, their effects on plant metabolism remain unclear. In contrast to the fungicides which directly affect pathogen metabolism, benzo(1,2,3) thiadiazole-7-carbothioic acid S-methylester (BTH) induces resistance against wheat pathogens by the activation of systemic acquired resistance in the host plant. In this study, we monitored gene expression in spring wheat after treatment with each of these agrochemicals in a greenhouse trial using a microarray containing 600 barley cDNA clones. Defence-related genes were strongly induced after treatment with BTH, confirming the activation of a similar set of genes as in dicot plants following salicylic acid treatment. A similar gene expression pattern was observed after treatment with fenpropimorph and some defence-related genes were induced by azoxystrobin, demonstrating that these fungicides also activate a defence reaction. However, less intense responses were triggered than with BTH. The same experiments performed under field conditions gave dramatically different results. No gene showed differential expression after treatment and defence genes were already expressed at a high level before application of the agrochemicals. These differences in the expression patterns between the two environments demonstrate the importance of plant growth conditions for testing the impact of agrochemicals on plant metabolism.

*Abbreviations:* BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methylester; INA, 2,6-dichloroisonicotinic acid, JA, jasmonic acid; SA, salicylic acid; SAR, systemic acquired resistance

## Introduction

Plants have evolved effective resistance mechanisms that enable them to defend against pathogen attacks. Nevertheless, all crops are susceptible to a number of major fungal pathogens that cause up to 20% of yield losses (Gullino *et al.*, 2000). In cereals, rusts, mildews and Septoria are the most damaging fungal diseases. In the last decades, a number of systemic fungicides with different modes of action and targets have been developed to reduce the losses caused by these diseases.

Strobilurins form a family of broad-spectrum fungicides that are derived from a natural compound, strobilurin A, which is produced by the wood-rotting fungus *Strobilurus tenacellus* (Bartlett *et al.*, 2002). The synthesis of derivatives of this molecule has led to several active compounds,

including azoxystrobin (Gullino *et al*., 2000). Azoxystrobin and the other strobilurins are inhibitors of fungal mitochondrial respiration by blocking the electron transfer at the $Q_0$ site of cytochrome $bc_1$ (Affourtit *et al*., 2000). Strobilurins currently represent 10% of the fungicide market and are used by farmers to control fungal pathogens such as powdery mildew and rusts. Besides their anti-fungal action, strobilurins are also known for their "greening effect" on the crop which is defined as a delayed leaf senescence and an increased grain-filling period (Bartlett *et al*., 2002). This side effect seems to result from the inhibition of ethylene biosynthesis by reduction of production of superoxide which is the mediator of the conversion reaction of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Wu and von Tiedemann, 2001).

Morpholines are another family of systemic fungicides, known since 1965 (Mercer, 1991). Fenpropimorph was discovered in 1979 and is still commonly used against mildews and rusts. It inhibits two enzymes of fungal sterol biosynthesis (Engels *et al*., 1998). The morpholine compound inhibits the enzymes sterol $\Delta^{14}$ reductase and $\Delta^8$–$\Delta^7$ isomerase by binding tightly to their catalytic site (Mercer, 1993; Debieu *et al*., 2000). Some phytotoxic effects like growth delay and altered phytosterol composition have been observed in cereals treated with this fungicide (Mercer *et al*., 1989; Khalil and Mercer, 1991).

Systemic acquired resistance (SAR) was discovered several decades ago and has been studied intensively (Metraux, 2001). A SAR response leads to pathogen resistance in the whole plant after biological or chemical stimulation. This mechanism allows the plant to protect itself against numerous viral, bacterial or fungal pathogens, depending on the species (Oostendorp *et al*., 2001). The two main chemical SAR enhancers are 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid S-methylester (BTH), which are both structurally similar to salicylic acid SA (Görlach *et al*., 1996). In dicotyledonous plants, these molecules induce pathogenesis-related genes and specific genes involved in signalling. Salicylic acid plays a key-role in the signal transduction pathway leading to SAR (Lawton *et al*., 1995). However, in monocotyledonous plants, the role of SA has not been clearly demonstrated, although the synthesis of SA is induced by aphid damage in barley (Chaman *et al*., 2003). In wheat, BTH can induce resistance to powdery mildew (*Blumeria graminis*), leaf rust (*Puccinia triticina*) and *Septoria* leaf spot (Görlach *et al*., 1996) but not to *Fusarium* head blight (Yu *et al*., 2001). The treatment of wheat plants with SA results in a lower resistance level against powdery mildew compared to plants treated with BTH, suggesting the involvement of other signalling pathway(s) to induce this SAR-like response (Görlach *et al*., 1996).

The putative effect of these compounds on crops has been tested by studying possible consequences of their primary action (for example with the measurement of sterol content after morpholine treatment (Khalil and Mercer, 1991)) and by few bioassays (Grossmann and Retzlaff, 1997). However, little is known on their effect on the whole plant metabolism. Genome-wide expression profiling (Schena *et al*., 1995) is a technology for studying changes of global gene expression after a specific treatment of the plant. For the two closely related species wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.), more than 950,000 ESTs have been characterised (see the Triticeae EST database website http://wheat.pw.usda.gov/genome/). Such collections of ESTs can be used for the construction of cDNA microarrays. The putative role of genes of unknown function can be predicted from similarity of expression patterns, even from different species (van Noort *et al*., 2003). In cereals, this technique has been used in only few studies. Rice cDNA microarrays have been used to study gene expression related to salt stress tolerance of rice (Kawasaki *et al*., 2001) and iron deficiency in barley (Negishi *et al*., 2002). Maize development and the response to UV radiation were studied with maize cDNA microarrays (Lee *et al*., 2002; Casati and Walbot, 2003). In barley, expression profiling was used for the detection of mutated genes (Zakhrabekova *et al*., 2002) and responses to drought and salt stress (Ozturk *et al*., 2002) or studying gene-for-gene interaction with powdery mildew using the Barley1 GeneChip from Affymetrix (Caldo *et al*., 2004).

Extensive toxicological risk assessment on the active compounds of pesticides and on their catabolites in the plant is an integral part of the regulatory approval process of pesticides and a huge database on these compounds is available at World Health Organisation (http://www.who.int/pcs/jmpr/jmpr.htm). Most of the studies concern food safety issues and human health. Therefore, a high food safety level of pesticide residues and

their metabolites in crops can be assumed. There is also a broad knowledge on endogenous plant compounds affecting human health (Stegelmeier *et al.*, 1999). In contrast, studies on possible changes in plant metabolism upon pesticide treatment are not mandatory and there is a lack of information on this aspect for most pesticides.

In order to determine whether the three compounds azoxystrobin, fenpropimorph and BTH alter wheat gene expression, we produced a cDNA microarray containing 600 barley genes covering the major plant biochemical pathways. Here, we report the impact of the two fungicides and the SAR enhancer on gene expression in wheat plants grown under controlled greenhouse conditions and compare these results to a similar trial where plants were grown in an agricultural environment.

## Materials and methods

### *Plant material and treatments*

Seeds of spring wheat (*Triticum aestivum* L., variety Greina) were grown in the greenhouse (16 h light/20 °C, 8 h night/16 °C, 2–4 seeds per pot). At growth stage 32 (Tottman, 1987), plants were treated with BTH (Bion®, Novartis (Basel, Switzerland) 60 g/ha, as recommended by the manufacturer). The second application of Bion® and the spraying of the two fungicides (azoxystrobin, Amistar® from Syngenta (Basel, Switzerland) and fenpropimorph, Corbel® from BASF (Wädenswil, Switzerland) at the concentration of 1 l/ha) were made at growth stage 39 (Tottman, 1987). Other plants were kept as untreated controls. In the field, the plants were sown in 5-row plots (1.3 m wide, 1.2 m long, approximately 50 seeds/row) near Zürich, Switzerland, at the Swiss Federal Research Station for Agroecology and Agriculture (FAL Reckenholz, 440 m above sea level). For each treatment, four plots were sprayed with one fungicide each, following the same protocol as for the treatment in the greenhouse. Four further plots were left untreated and used as control. For both trials, flag leaves were harvested at 24 h, 1 and 2 weeks after treatment.

### *Preparation of the cDNA microarray*

A total of 600 cDNA clones of barley (*Hordeum vulgare* L., varieties Morex and CI16155) from our laboratory (SFR clones) and from Clemson University (HVSMEg, HVSMEh and HV_Ceb clones) were chosen to cover major biochemical pathways (for more details about the libraries, see http://wheat.pw.usda.gov/genome for our laboratory's library and http://www.genome.clemson.edu/projects/barley for the Clemson University collection). We used a method adapted from Reymond *et al.* (2000) to print PCR products amplified from these clones and negative controls (human and *Arabidopsis thaliana* cDNA) onto coated glass slides. Each clone was printed twice. The Clemson clones were amplified twice in 150 $\mu$l with Taq polymerase (Sigma-Aldrich, Buchs, Switzerland) using 5′ end amino-modified M13 universal primers in 35 cycles (94 °C, 45 s; 52 °C, 45 s; 72 °C, 90 s). The SFR clones were amplified in the same volume with the 5′ end amino-modified TriplExAmp primers (94 °C, 45 s; 62 °C, 45 s; 72 °C, 90 s) for 10 cycles followed by 25 cycles (94 °C, 45 s; 55 °C, 45 s; 72 °C, 90 s). The DNA products were checked on agarose gels and sequenced with a 377 ABI prism™ DNA sequencer, to confirm their identity. They were concentrated during purification with multiscreen-PCR (Millipore, Volketswil, Switzerland). Two sets of slides were produced, using two different DNA spotters. In the first set, cDNA samples were diluted in print buffer (NoAb Biodiscoveries Inc., Mississauga, Canada) at a concentration of 0.5–1 $\mu$g/$\mu$l. PCR products were printed using a GMS417 Arrayer (Affymetrix, Santa Clara, USA) on epoxy-coated glass slides (NoAb Biodiscoveries Inc.). The second set was produced according to the protocol of P. Reymond (http://www.unil.ch/ibpv/WWWPR/Docs/protocols.htm), using the printing facilities of Lausanne University (printing robot (OmniGrid), GeneMachines, Ann Arbor, USA). The purified probes were diluted with a 2 × spotting solution (6 × SSC, 3 M betain) to the final concentration of 0.5–1 $\mu$g/$\mu$l DNA, 3 × SSC, 1.5 M betain and then printed on QMT Aldehyde slides (Peqlab Biotechnologie Gmbh, Erlangen, Germany).

### *RNA isolation and preparation of the fluorescent targets*

For each treatment and time point, several flag leaves were pooled to reduce biological variation. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Basel, Switzerland), and 40 $\mu$g of RNA was used for the reverse-tran-

scription, using either Cy5-labelled dCTP for the treated samples or Cy3-dCTP (Amersham Biosciences, Otelfingen, Switzerland) for the non-treated control samples, adapted from Reymond *et al.* (2000). The reaction was incubated for 2 h at 42 °C. After pooling the treated and control samples, RNA was degraded, the labelled cDNAs were purified using the MinElute PCR purification kit (Qiagen, Basel, Switzerland) and diluted in hybridisation solution (3× SSC, 0.2% SDS, 0.2% yeast tRNA).

### *Hybridisation on microarrays, scanning and analysis*

The target solution was denatured for 1 min at 95 °C and applied to the microarray slide which was then covered with a cover slip. Hybridisations were performed in chambers placed in a water bath at 65 °C for 14–16 hr. The slides were washed twice in 1 × SSC, 0.03% SDS for 6 min, then twice in 0.2 × SSC for 5 min and finally twice in 0.05 × SSC for 5 min. They were subsequently dried by centrifugation.

Scanning of the microarray slides was performed using a ScanArray 5000 (PerkinElmer Life Sciences, Rodgau – Jügesheim, Germany) at the resolution of 10 $\mu$m/pixel. Photomultiplier and laser power settings were adjusted in order to obtain similar intensity levels of signal for the control spots (house-keeping genes and alien cDNA, Stratagene, Amsterdam, Netherlands) for both channels. Pictures were analysed by Imagene 4.1 software (BioDiscovery Inc., Los Angeles, USA). Spots flagged as empty by the software or manually were removed from the analysis. Normalisation of the signal intensities between the two channels was performed using the global method, and between the slides by scale normalisation (Yang *et al.*, 2002). Experiments with reversion of the dyes did not show significant differences in hybridisation level, so only one way of labelling (control with Cy3 and treated sample with Cy5 dye) was further used. Hybridisations were performed in triplicates, using three different samples treated by the same compound for the three time points. To better assess the gene expression of untreated plants from both growth conditions, microarrays were also performed for the three time points. The samples from the greenhouse were labelled with the Cy5 and those from the field with Cy3. Genes were considered induced or repressed by Significance Analysis of Microarray (SAM, Excel Add-in available at http://www-stat.stanford.edu/~tibs/SAM/). This program allowed the determination of both differentially expressed genes and corresponding false discovery rates (FDR, (Tusher *et al.*, 2001; Hu *et al.*, 2003)). The number of differentially expressed genes and the FDR were determined in order to have one gene considered as falsely detected (Samimi *et al.*, 2005). The obtained FDR were generally below 10% in the greenhouse but higher when there were very few genes differentially expressed after the treatments. Therefore, when only 3–6 genes out of the 600 genes of the chip were differentially expressed, the FDR were between 16–37.5%. Such high ratios have already been observed in a recent study on plant–insect interactions where few genes were differentially expressed and high FDR were obtained (Reymond *et al.*, 2004).

Cluster analyses were carried out using Genesis software (Sturn *et al.*, 2002). Reproducibility between the replicates was checked by measuring correlation and data are presented in trees created with Genesis software.

### *Northern blot analysis*

The results from microarray experiments were partially validated by RNA blot analysis. The same quantity of RNA (40 $\mu$g) was electrophoresed and transferred to a nylon membrane as previously described (Feuillet *et al.*, 1997). The labelled probes *WCI2*, *5*, *WIR1c*, actin, *PR 1a*/*1b* (HV_CEb0006J08f)) were prepared using standard procedures (Sambrook *et al.*, 1989) with clones previously used as templates for the barley cDNA microarray. The RNA blots were analysed using Biomax MS-1 film (Kodak, Lausanne, Switzerland).

## Results

### *Barley cDNA microarray design*

To study gene expression in wheat, we made a cDNA microarray containing 600 cDNAs. Barley and wheat are two species showing high similarity of their gene order (Feuillet and Keller, 2002) and high conservation of gene sequences between the two

species (Bennetzen *et al.*, 1998). Several studies have shown that coding sequence identity can reach 100% and is usually over 90% (Ramakrishna *et al.*, 2002; SanMiguel *et al.*, 2002; Caldwell *et al.*, 2004). Therefore, there is good cross-hybridisation between nucleic acids of barley and wheat. Furthermore, as the probes are longer than 400 bp, the cDNA microarray allows small inter-species differences with only minimal consequences on hybridisation (Adjaye *et al.*, 2004; Close *et al.*, 2004). The genes used for our chip were chosen from barley (*Hordeum vulgare* cv. Morex) cDNA libraries from pre-anthesis spikes, spikes 5–45 days after pollination, plants challenged with the powdery mildew pathogen (HVSMEg clones, HVSMEh clones and HV_Ceb clones, Clemson University, respectively) and from our laboratory collection (SFR clones). In blast analyses, we found that the 600 sequences all had corresponding ESTs in the wheat libraries (E value between 0 and e-20 and mean identity level of 90%), reflecting the high homology level between the barley and wheat sequences (see supplementary Table 6). Expression of genes encoding enzymes from the major biochemical pathways of primary and secondary metabolism was analysed. In preliminary tests, wheat derived labelled samples resulted in successful cross-hybridisation with the barley probes on our microarray slides (data not shown). This is in agreement with the generally observed high conservation of coding sequences in wheat and barley (Goff *et al.*, 2002; Close *et al.*, 2004).

### *Effect of the BTH treatment*

The analysis of BTH treated plants grown in the greenhouse revealed that 17 genes were differentially regulated after 24 h (false discovery rate FDR 5.8%), 24 after 1 week (FDR 4.1%) and 9 after 2 weeks (FDR 8.9%), which represents about 5% of the genes present on the chip. All of these differentially expressed genes were over-expressed and none repressed (Figure 1, Tables 1–3). The differentially regulated transcripts mainly belong to defence-related genes. Genes encoding glucanase, lipoxygenase and pathogenesis-related genes (*PR1a/1b, 2, B1*), the wheat induced resistance genes (*WIR1B*, *1C* and *232*) and the wheat chemically induced genes 2 (*WCI2*, lipoxygenase), 1 (*WCI1*, jasmonate-induced), 4 (*WCI4*, protease) and 5 (*WCI5*) showed an increased gene expression

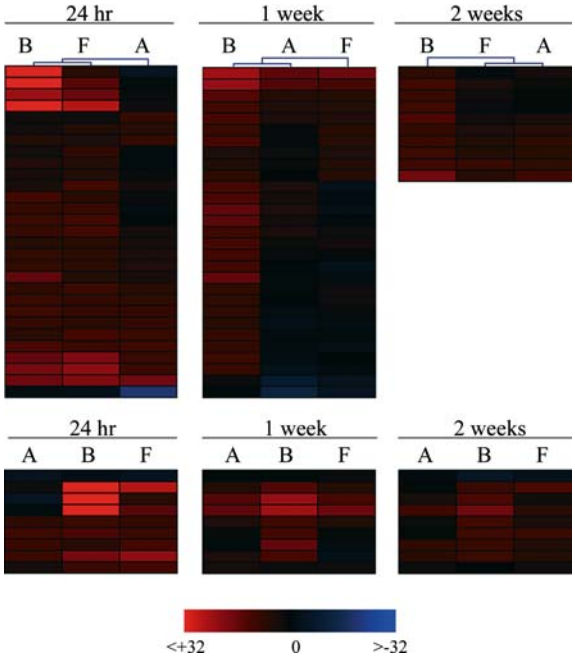


*Figure 1.* Analysis of gene expression after chemical treatments. Hierarchical clustering of the differentially expressed genes, as displayed by the software Genesis, is shown after treatment with azoxystrobin (A), BTH (B) and fenpropimorph (F) in the greenhouse at the time points 24 h, 1 and 2 weeks after treatment. The colour scale bar represents the ratio values. Genes with higher expression level after treatment appear in red; those with lower hybridisation intensity appear in blue. After 24 h, the expression patterns of BTH-treated and fenpropimorph-treated plants clustered together (blue tree on top of the clusters). For the subsequent time point, expression profiles of azoxystrobin-treated and BTH-treated plants were more similar to each other, and after 2 weeks, treatments with fenpropimorph and azoxystrobin resulted in similar differentially expressed genes. The lower panel shows the microarray results of the subgroup of nine genes also analysed by Northern blot (Figure 2). From top to bottom: actin gene, the lipoxygenase gene (*WCI2*), the thiol protease gene (*WCI4*), the wheat chemically induced 1 and 5 genes, a wheat induced resistance gene (*WIR1c*), the pathogenesis-related genes *PR 1* (HV_CEb0010L20f) and *PR 1a/1b* (HV_CEb0006J08f) and the protein disulfide-isomerase gene (HVSMEg0005I10f).

of 2- to nearly 60-fold 24 h after treatment when compared to untreated plants (Table 1). Most of these genes remained over-expressed 1 and 2 weeks after treatment but with decreasing over-expression ratios compared to those obtained at 24 h (Tables 2 and 3). These results were confirmed for some genes (*PR1, PR1a/1b*, *PDI, WCI1, WCI2*, *WCI4, WCI5* and *WIR1c*) by Northern blot analysis (Figure 2) and are in agreement with other studies (Görlach *et al.*, 1996; Stadnik and Buchenauer, 1999). Genes

*Table 1.* Differentially expressed genes in the greenhouse trial 24 h after treatment with either BTH (B), fenpropimorph (F) or azoxystrobin (A).

| Gene ID | Accession number | Putative function | B 24 h | F 24 h | A 24 h |
|---|---|---|---|---|---|
| wci2 | TAU32428 | UP\|Q41520 (Q41520) Lipoxygenase (Fragment) (EC 1.13.11.12) | **56.7** | **17.8** | 1.3 |
| wci1 | TAU32427 | PIR\|T06273 Benzothiadiazole-induced protein clone WCI-1 - wheat | **44.3** | **4.7** | 0.9 |
| wci4 | TAU32430 | homologue to UP\|Q41522 (Q41522) Thiol protease | **35.0** | 2.2 | 0.6 |
| HVSMEh0095N14f | BE455009 | UP\|LOX1_HORVU (P29114) Lipoxygenase 1 (EC 1.13.11.12) | **12.7** | **6.1** | 1.2 |
| HV_CEb0006J08f | BE215358 | UP\|PR1A_HORVU (P32937) Pathogenesis-related protein 1A/1B precursor | **7.3** | **10.7** | **3.0** |
| HV_CEb0024H14f | BE559397 | UP\|PR12_HORVU (P35792) Pathogenesis-related protein PRB1-2 precursor | **6.1** | **8.2** | **6.5** |
| wir232 | TATHAU | UP\|Q94F70 (Q94F70) Putative thaumatin-like protein | **5.5** | **8.2** | **2.8** |
| HVSMEg0016C06f [a] | BG344787 | similar to UP\|COPD_ORYSA (P49661) Coatomer delta subunit | **5.3** | 1.9 | 1.6 |
| wir1c | TARNAWIR1 | UP\|Q41581 (Q41581) WIR1 protein | **3.9** | **3.0** | **3.2** |
| HV_CEb0010H13f | BE216428 | similar to UP\|Q8H841 (Q8H841) Putative receptor-like protein kinase | **3.5** | **2.2** | 1.2 |
| HV_CEb0010G19f | BE216411 | UP\|E13B_HORVU (P15737) Glucan endo-1 3-beta-glucosidase GII precursor | **3.1** | **2.7** | 2.7 |
| HV_CEb0003K12f | BE214507 | UP\|P93180 (P93180) Pathogenesis-related protein 4 precursor | **3.1** | **3.6** | 1.0 |
| wci5 | TAU32431 | PIR\|T06278 Benzothiadiazole-induced protein clone WCI-5 - wheat | **3.0** | **2.7** | **2.5** |
| HVSMEg0005I10f | BG343356 | UP\|PDI_HORVU (P80284) Protein disulfide-isomerase precursor (PDI) (Endosperm protein E-1) | **2.8** | **3.5** | 1.5 |
| wir1b | WHTWIR1PR | UP\|WIRB_WHEAT (Q01481) WIR1B protein | **2.8** | **2.3** | **2.7** |
| HVSMEg0013J19f | BE060855 | UP\|LX23_HORVU (Q8GSM2) Lipoxygenase 2.3 chloroplast precursor (LOX2:Hv:3) (EC 1.13.11.12) | 2.7 | **2.9** | 0.8 |
| HVSMEh0088H07f | BE195158 | UP\|PDI_HORVU (P80284) Protein disulfide-isomerase precursor (PDI) (Endosperm protein E-1) | **2.6** | 2.3 | 1.6 |
| HV_CEb0002C16f | BE214080 | UP\|ENPL_HORVU (P36183) Endoplasmin homolog precursor | **2.6** | 2.2 | 1.8 |
| HV_CEb0003J11f | BE214483 | UP\|Q43765 (Q43765) Chitinase (EC 3.2.1.14) | **2.5** | **3.0** | **2.2** |
| HV_CEb0010L20f | BE216529 | UP\|PR1_HORVU (Q05968) Pathogenesis-related protein 1 precursor | 2.1 | **3.6** | 2.9 |
| HV_CEb0017B21f | BE558296 | similar to UP\|Q7XH17 (Q7XH17) Putative receptor-like protein kinase 4 | 2.1 | **2.2** | **1.5** |
| HV_CEb0003A03f | BE214285 | similar to GP\|13897320 Somatic embryogenesis receptor-like kinase 2 {*Zea mays*} | 1.9 | 1.7 | **2.7** |
| HV_CEb0021J19f | BE519892 | similar to UP\|Q8H8H7 (Q8H8H7) Putative flavanone 3-hydroxylase | 1.9 | **2.0** | 0.9 |
| HVSMEg0011M01f | BE060255 | UP\|Q9M4C7 (Q9M4C7)Allene oxide synthase (EC 4.2.1.92) | 1.6 | **3.4** | 1.4 |
| HVSMEg0003M07f | AW982621 | UP\|Q6RYF4 (Q6RYF4) Coatomer alpha subunit | 1.6 | **2.0** | 1.2 |
| HV_CEb0003D01f | BE214349 | UP\|O65189 (O65189) Glucan endo-1 3-beta-glucosidase | 1.4 | **2.7** | 0.9 |
| HV_CEb0003A01f | BE214283 | UP\|Q43764 (Q43764) Chitinase (EC 3.2.1.14) | 1.4 | **1.9** | 2.1 |
| HVSMEh0100J22f [a] | BG418805 | homologue to UP\|FKB7_WHEAT (Q43207) 70 kDa Peptidylprolyl isomerase | 1.4 | 1.2 | **2.7** |
| HV_CEb0003P20f | BE214619 | UP\|CHS1_HORVU (P26018) Chalcone synthase 1 | −1.2 | −1.3 | **−9.0** |

[a]Clones that have not given the same sequencing result as in the database of Clemson University.
Intensity ratios of genes determined to be differentially expressed by SAM analysis are in bold type. The positive values indicate gene induction and negative values indicate gene repression. The FDR were 5.8%, 4.5% and 10% for B, F and A, respectively.

*Table 2.* Differentially expressed genes in the greenhouse trial 1 week after treatment with either BTH (B), fenpropimorph (F) or azoxystrobin (A).

| Gene ID | Gene accession | Putative function | B 1 week | F 1 week | A 1 week |
|---|---|---|---|---|---|
| wci1 | TAU32427 | PIR\|T06273 Benzothiadiazole-induced protein clone WCI-1 - wheat | **13.2** | **6.3** | **5.1** |
| wci4 | TAU32430 | Homologue to UP\|Q41522 (Q41522) Thiol protease | **11.7** | **3.8** | **4.4** |
| HV_CEb0010L20f | BE216529 | UP\|PR1_HORVU (Q05968) Pathogenesis-related protein 1 precursor | **5.4** | −1.2 | −1.1 |
| HV_CEb0024H14f | BE559397 | UP\|PR12_HORVU (P35792) Pathogenesis-related protein PRB1-2 precursor | **5.3** | −1.3 | 1.8 |
| wir232 | TATHAU | UP\|Q94F70 (Q94F70) Putative thaumatin-like protein | **4.9** | −1.1 | 1.4 |
| wci2 | TAU32428 | UP\|Q41520 (Q41520) Lipoxygenase (Fragment) (EC 1.13.11.12) | **4.5** | 2.0 | 2.0 |
| HV_CEb0003K12f | BE214507 | UP\|P93180 (P93180) Pathogenesis-related protein 4 precursor | **3.9** | −1.2 | 1.5 |
| HV_CEb0010N06f | BE216563 | Homologue to GP\|17981573 Kinase R-like protein {*Triticum aestivum*} | **3.8** | 2.1 | 1.1 |
| HV_CEb0006J08f | BE215358 | UP\|PR1A_HORVU (P32937) Pathogenesis-related protein 1A/1B precursor | **3.8** | −1.3 | 1.7 |
| HVSMEh0095N14f | BE455009 | UP\|LOX1_HORVU (P29114) Lipoxygenase 1 (EC 1.13.11.12) | **3.7** | 1.8 | 1.7 |
| HVSMEg0002G09f | AW982228 | Homologue to UP\|Q75RZ2 (Q75RZ2) Putative caffeoyl CoA O-methyltransferase | **3.7** | 1.4 | 1.2 |
| HV_CEb0021J19f | BE519892 | similar to UP\|Q8H8H7 (Q8H8H7) Putative flavanone 3-hydroxylase | **3.5** | −1.4 | −1.0 |
| wir1c | TARNAWIR1 | UP\|Q41581 (Q41581) WIR1 protein | **3.5** | 1.1 | 1.1 |
| wci5 | TAU32431 | PIR\|T06278 Benzothiadiazole-induced protein clone WCI-5 - wheat | **3.3** | 1.9 | 1.8 |
| HV_CEb0010G19f | BE216411 | UP\|E13B_HORVU (P15737) Glucan endo-1 3-beta-glucosidase GII precursor | **3.3** | 1.3 | 1.6 |
| HV_CEb0011F02f [a] | BE216529 | UP\|PR1_HORVU (Q05968) Pathogenesis-related protein 1 precursor | **3.0** | −1.0 | −1.2 |
| HV_CEb0015B10f | BE558194 | weakly similar to GP\|22535653 Putative protein kinase Xa21 receptor type precursor {*Oryza sativa*} | **3.0** | **2.3** | 1.1 |
| HV_CEb0011F16f | BE216724 | Homologue to UP\|Q8W4U9 (Q8W4U9) Clathrin assembly protein AP17-like protein | **2.9** | −1.1 | −1.4 |
| HV_CEb0011I04f | BE216768 | similar to UP\|Q84S61 (Q84S61) Putative serine/threonine kinase protein | **2.7** | −1.1 | −1.1 |
| HVSMEh0088K24f | BE195244 | UP\|Q42839 (Q42839) Chitinase (EC 3.2.1.14 ) | **2.6** | −1.2 | −1.1 |
| HV_CEb0010N04f | BE216561 | Homologue to UP\|Q41328 (Q41328) Pto kinase interactor 1 | **2.5** | 1.3 | 1.0 |
| HV_CEb0017B21f | BE558296 | similar to UP\|Q7XH17 (Q7XH17) Putative receptor-like protein kinase 4 | **2.4** | −0.9 | −1.2 |
| HVSMEg0007A12f | BG343889 | similar to UP\|Q6J2K7 (Q6J2K7) Protein tyrosine phosphatase | **2.4** | −0.8 | −1.0 |
| HVSMEh0099N09f [a] | BE601871 | Homologue to UP\|TBP2_WHEAT (Q02879) TATA-box binding protein 2 | 2.3 | **2.1** | 1.0 |
| HV_CEb0010P06f | BE216610 | Homologue to UP\|Q43220 (Q43220) Peroxidase (EC 1.11.1.7) | **2.1** | −1.2 | −1.3 |
| HVSMEh0102L08f | BE603237 | similar to UP\|Q8S8Z0 (Q8S8Z0) Protein phosphatase 2C | 1.9 | **2.0** | 1.1 |
| HVSMEh0081G18f | BE193520 | similar to SP\|P37837 Transaldolase (EC 2.2.1.2) | 1.2 | −1.5 | **−2.5** |
| HVSMEh0081M04f | BE193575 | Homologue to GB\|CAA75793 Sucrose synthase 2 {*Hordeum vulgare* subsp. *vulgare*} | −1.1 | −1.6 | **−3.9** |

[a]Clones that have not given the same sequencing result as in the database of Clemson University.
Intensity ratios of genes determined to be differentially expressed by SAM analysis are in bold type. The positive values indicate gene induction and negative values indicate gene repression. The FDR were 4.1%, 16% and 25% for B, F and A, respectively.

*Table 3.* Differentially expressed genes in the greenhouse trial 2 weeks after treatment with either BTH (B), fenpropimorph (F) or azoxystrobin (A).

| Gene ID | Accession number | Putative function | B 2 weeks | F 2 weeks | A 2 weeks |
|---|---|---|---|---|---|
| wci1 | TAU32427 | PIR\|T06273 Benzothiadiazole-induced protein clone WCI-1 - wheat | **6.4** | **2.2** | **3.3** |
| wci4 | TAU32430 | homologue to UP\|Q41522 (Q41522) Thiol protease | **4.0** | 1.4 | 1.6 |
| wci5 | TAU32431 | PIR\|T06278 Benzothiadiazole-induced protein clone WCI-5 - wheat | **3.4** | 1.6 | 1.2 |
| wir1c | TARNAWIR1 | UP\|Q41581 (Q41581) WIR1 protein | **3.2** | 1.1 | 1.0 |
| wir232 | TATHAU | UP\|Q94F70 (Q94F70) Putative thaumatin-like protein | **3.2** | **2.9** | **2.4** |
| HV_CEb0006J08f | BE215358 | UP\|PR1A_HORVU (P32937) Pathogenesis-related protein 1A/1B precursor | **3.1** | 1.9 | 2.2 |
| HV_CEb0010L20f | BE216529 | UP\|PR1_HORVU (Q05968) Pathogenesis-related protein 1 precursor | **2.9** | 1.9 | 2.5 |
| wci2 | TAU32428 | UP\|Q41520 (Q41520) Lipoxygenase (Fragment) (EC 1.13.11.12) | 2.9 | **3.9** | **2.7** |
| HV_CEb0024H14f | BE559397 | UP\|PR12_HORVU (P35792) Pathogenesis-related protein PRB1-2 precursor | **2.7** | 1.7 | 2.1 |
| pBTag | AJ237942 | UP\|Q9SM34 (Q9SM34) Putative germin-like protein precursor | **2.4** | −1.1 | 1.4 |

Intensity ratios of genes determined to be differentially expressed by SAM analysis are in bold type. The positive values indicate gene induction and negative values indicate gene repression. The FDR were 8.9%, 20% and 37.5% for B, F and A, respectively.

belonging to other functional classes were also up-regulated. Two protein disulfide isomerase genes and one coatomer protein (COP) subunit gene showed an induction of RNA synthesis after 24 h, suggesting an increased biosynthesis of secreted or cell surface proteins (Harter, 1995; Ciaffi *et al.*, 2001). Genes encoding the putative proteins flavanone-3-hydroxylase and caffeoyl CoA *O*-methyltransferase, involved in flavonoid biosynthesis, showed an activation of transcription only after one week.

### *Impact of the fenpropimorph treatment*

After fenpropimorph treatment, the overall expression pattern was similar to the one obtained after BTH treatment (Figure 1) although seven




*Figure 2.* RNA blot analysis showing differential expression 24 h, 1 and 2 weeks after fungicide treatment in the greenhouse trial. C: non-treated control, A: azoxystrobin, B: BTH, F: fenpropimorph. Nine labelled probes were used: the actin gene as quality control of the blots, a lipoxygenase gene (*WCI2*), three wheat chemically induced genes (*WCI4*, *WCI1* and *WCI5*), a wheat induced resistance gene (*WIR1c*), the pathogenesis-related genes *PR 1* (HV_CEb0010L20f) and *PR1a*/*1b* (HV_CEb0006J08f), and the protein disulfide-isomerase gene (HVSMEg0005I10f).

additional genes (the allene oxide synthase, the putative flavanone 3-hydroxylase, four *PR* and one putative kinase genes) were significantly over-expressed 24 h after fenpropimorph treatment (FDR 4.5%) compared to the BTH-treated plants (Table 1). For *WCI2* and *1*, the treated/control ratios were lower than the ones obtained with BTH, suggesting a weaker impact of this compound on plant metabolism compared to the SAR enhancer (Tables 1–3). Defence-related genes such as the glucanase and *PR* genes were induced after 24 h but did not show any differential expression later, suggesting a rapid but transient response. The putative thaumatin-like gene (*WIR232*) showed a different pattern, with an induction after 24 h, no differential expression after 1 week but again an increase of mRNA amount after 2 weeks compared to untreated plants. This gene seems to be involved in two phases of the reaction and could be regulated by a different pathway. Expression of the *PR1, PR1a/1b, WCI2, WCI4, WCI5* and *WIR1c* and *PDI* genes were also analysed by Northern blot analyses, giving results similar to the microarray hybridisations (Figure 2). Interestingly, the allene oxide synthase gene was up-regulated after 24 h, suggesting an increase of JA biosynthesis. This indicates that the JA synthesis would be induced after the treatment with fenpropimorph whereas it was not after BTH treatment. As the putative flavanone-3-hydroxylase gene is also induced, the flavonoid synthesis pathway could be triggered early after fenpropimorph treatment.

*Effect of the treatment with azoxystrobin*

The strobilurin fungicide is known to produce a "green effect" on wheat plants as non-fungicidal secondary effect, with darker green leaves, enhanced concentration of chlorophyll and increased biomass production (Grossmann and Retzlaff, 1997). It also induces some antioxidant activity (Wu and von Tiedemann, 2002). After azoxystrobin treatment, only few genes showed an alteration of their expression, with a high FDR of 10%, 16% and 37.5% as there were only ten, four and three genes differentially expressed after 24 h, 1 and 2 weeks, respectively (Tables 1–3). The *PR1, 1A/1B* and *B1-2* genes were significantly over-expressed after 24 h only, and the *WCI1* gene after 1 and 2 weeks. The expression levels of *WCI5, WIR1b* and *WIR1c* were similar to each other with a small up-regulation detected by microarray analysis only 24 h after treatment. No statistically significant induction of these genes was observed with microarray analysis for the last two time points. However, over-expression of *WCI5* and *WIR1c* was detected for all time points by Northern analysis (Table 1, Figure 2). Interestingly, the wheat chemically induced genes 4 and 2 that had the strongest up-regulation after BTH and fenpropimorph treatment were not induced after 24 h but showed up-regulation after 1 and 2 weeks, respectively. This indicates that the responses after the application of azoxystrobin are only partially overlapping with the pattern generated by the two other compounds.

A chalcone synthase gene showed a high repression level 24 h after treatment. This gene, like the lipoxygenase genes, is regulated by ethylene (Wan *et al*., 2002). The antioxidant and "green" effects of azoxystrobin are attributed to a reduction of ethylene production (Grossmann and Retzlaff, 1997). Thus, the reduction of chalcone synthase (HV_CEb0003P20f) expression as well as the absence of over-expression of the three lipoxygenase genes (*WCI2*, HVSMEg0013J19f and HVSMEh0095N14f) after 24 h confirm that azoxystrobin has a specific effect on wheat gene expression which is different from the action of fenpropimorph and BTH (Table 1). After 1 week, two genes involved in sugar metabolism (a gene similar to a transaldolase gene and a sucrose synthase 2 gene) were down-regulated.

*Impact on gene expression of the plant protection compounds in the field*

Fungicide and BTH treatments were made in the field to compare gene expression with plants treated in the greenhouse. Surprisingly, no gene showed differential expression after treatment of the plants in the field, whatever the compound used. The *WCI2* gene showed higher expression in the Northern blot 24 h after BTH treatment (Figure 3) but this was not statistically significant in the microarray experiments. The absence of differentially expressed genes in the field trial was supported by a high reproducibility between replicates (see supplementary Figure 4). The non-treated plants showed a high expression of the defence-related genes that were over-expressed

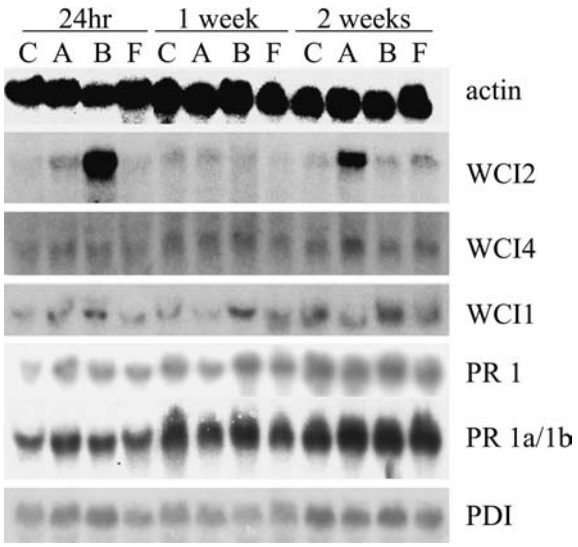


*Figure 3*. RNA blot analysis showing differential expression 24 h, 1 and 2 weeks after fungicide treatment in the field trial. C: non-treated control, A: azoxystrobin, B: BTH, F: fenpropimorph. Seven labelled probes were used: actin gene as quality control of the blots, a lipoxygenase gene (*WCI2*), and the wheat chemically induced genes (*WCI4*, *WCI1* and *WCI5*), the pathogenesis related genes *PR 1* (HV_CEb0010L20f) and *PR 1a/1b* (HV_CEb0006J08f). Twenty four hours after the BTH treatment and 2 weeks after the azoxystrobin treatment, the *WCI2* gene showed high expression but in microarray experiments this was not statistically significant and only found in one of the three replicate slides.

after the treatments in the greenhouse trial (Figure 3 and supplemental Table 4). For all three time points, the *PR1, PR1a/1b, WIR232, WIR1c*, a $\beta$1-3 glucanase, two peroxidase and four chitinase genes were significantly higher expressed compared to the plants grown in the greenhouse. Other defence-related genes were also more expressed in the field for one or two time points (*PR4, WIR1a,* $\beta$1-3 glucanase, glutathione peroxidase, two chalcone synthase, two kinase genes). These results suggest that many of the genes induced by these fungicides in the greenhouse are constitutively expressed during growth in the agricultural environment. However, not all the defence-related genes did behave similarly. The *WCI 1, 2* and *4* genes did not show any significant changes in expression between the two growth conditions (supplemental Table 4). The alteration of response to the plant protection compounds could be due to a combination of stresses occurring in the field (Rizhsky *et al*., 2002). In contrast to the genes over-expressed under field conditions, some genes like the RNase S-like protein gene or the inositol-3-phosphate synthase gene were more expressed in the greenhouse than in the field (supplementary Table 4). However, the apparent over-expression of the RNAse S-like gene could be due to the time-shift when collecting the samples (morning in the greenhouse and afternoon for the field experiments) as it has been shown that this gene is light responsive (Gausing, 2000).

## Discussion

A SAR enhancer and two commonly used fungicides with different and specific chemical modes of action were used in this study to better understand their impact on wheat gene expression and plant metabolism using cDNA microarrays. A relatively small proportion of the genes present on the chip (around 5%) showed differential expression after the treatments. Although few transcripts showed differential expression profiles, common and compound-specific patterns were observed after the three different treatments. Most of the differentially expressed transcripts detected belonged to defence-related gene families, such as the *PR* and *WIR* genes, revealing that fungicides can have not only an impact on their fungal targets but have also an impact on the plant itself more or less similar to BTH. Interestingly, no gene coding for key-enzymes of the primary metabolism showed differential expression pattern after BTH and fenpropimorph treatments, showing that these compounds mainly affect specific sets of genes and defence pathways. Only two genes belonging to the sugar metabolism pathway and coding for sucrose synthase 2 and transaldolase, respectively, were down-regulated 1 week after azoxystrobin treatment.

BTH, fenpropimorph and azoxystrobin induced genes known to be involved in plant defence against pathogens. The *PR1* and wheat induced resistance genes (*WIR1b, WIR1c, 232*) were activated early after the treatment with these compounds, following a similar pattern as in *Arabidopsis* and tobacco after BTH treatment (Friedrich *et al*., 1996; Lawton *et al*., 1996). However, the induction of the *PR1* genes after BTH treatment contradicts previous results (Molina *et al*., 1999; Yu *et al*., 2001) where no activation of these genes was observed, but is in agreement with the results of Görlach *et al*. (1996). Thus, the induction of the

*PR1* genes after stimulation by BTH seems to depend on wheat variety, developmental stage or growth conditions of the plant.

After BTH treatment, the wheat chemically induced genes (*WCI2, WCI1, WCI4* and *WCI5*) were strongly induced as previously described (Rebmann *et al*., 1991; Bull *et al*., 1992; Görlach *et al*., 1996; Mauch *et al*., 1997). These genes were also induced after the application of fenpropimorph although the levels of differential expression were not as high as after the BTH treatment. In addition, genes like the PDI and COP genes were also induced after BTH and fenpropimorph treatments. Their expression in wheat has been described during plant development (Ciaffi *et al*., 2001) but not after chemical treatment. These results might indicate that BTH and fenpropimorph induce the secretory and cell surface protein biosynthesis machinery. The activation of the PDI genes could play a role in signal transduction, as the enzyme can break disulfide bonds that can subsequently allow the monomerisation of a key component of this pathway, as described for NPR1 in *Arabidopsis* (Mou *et al*., 2003).

In dicotyledons, SA plays an essential role in pathogen resistance as plants defective in SA synthesis cannot develop a SAR response (Lawton *et al*., 1995). However, in monocots, SA is probably less important, as treatment with this molecule only resulted in mild resistance against fungal pathogens and low induction of genes that are over-expressed after BTH application (Görlach *et al*., 1996). In our experiment, the ethylene and jasmonate pathways seem to be important for triggering SAR as the *WCI2* and *WCI1* genes (encoding lipoxygenase and jasmonate-induced protein, respectively) showed the highest level of over-expression after BTH and fenpropimorph applications.

Interestingly, the morpholine fungicide qualitatively induced a very similar expression pattern in wheat as BTH, albeit at a quantitatively lower level. Furthermore, the changes induced by this sterol biosynthesis inhibitor were more transient, as fewer differentially expressed genes were observed after 1 and 2 weeks. The allene oxide synthase gene was over-expressed after 24 h. This gene encodes one of the key enzymes of jasmonic acid synthesis. This molecule could enhance the defence responses via an increase of the phenylpropanoid pathway metabolism. As morpholine seems to have a relatively mild effect on gene expression, the previously observed negative growth effects on plants (Mercer *et al*., 1989) could be due to the inhibition of major sterol biosynthesis or to a repression of the soil microorganisms after treatment with fenpropimorph (Thirup *et al*., 2001).

The azoxystrobin treatment slightly increased the expression of *PR* genes after 24 h but not the expression of lipoxygenase genes that are known to be induced by ethylene. Furthermore, and in contrast to the other two treatments, down-regulation of the chalcone synthase gene was observed 24 h after the treatment. This gene is activated by ethylene and its corresponding protein is involved in the biosynthesis of phytoalexins. The lack of expression of this gene might indicate that ethylene synthesis and/or signalling is repressed after azoxystrobin treatment (Schenk *et al*., 2000). Thus, lower ethylene levels could explain the lower induction of defence-related genes after azoxystrobin treatment compared to the results obtained after the BTH and fenpropimorph treatment.

Our data demonstrate that the morpholin fungicide obviously not only acts on the pathogen metabolism (Engels *et al*., 1996; Rohel *et al*., 2001) but can also lead to the induction of a similar set of plant defence genes as the SAR enhancer BTH. Such secondary effects of fungicides might be beneficial for the plants by inducing defence mechanisms before pathogen attack. The effectiveness of another fungicide, fosetyl, is dependent on the SAR pathway in *A. thaliana* and this compound may trigger the SAR because of its phytotoxicity (Molina *et al*., 1998). The same phenomenon could also explain the induction of the defence-related genes after the application of morpholin on wheat. The plant defence response and the direct fungicidal activity might both synergistically contribute to the observed action of morpholin against pathogens (Molina *et al*., 1998). The azoxystrobin treatment appears to have a weaker impact on defence-related gene expression because of ethylene inhibition but still some slight induction of *PR* genes occurred, probably due to other signalling molecules. The lower ethylene level could affect the defence response of the plant and could explain the lower fungicidal activity of azoxystrobin against some wheat pathogens if compared to other compounds

(McCabe *et al*., 2001). Similarly, strobilurin was also shown to induce resistance against several pathogens in tobacco but reduced the hypersensitive response (Herms *et al*., 2002). In parallel, Arabidopsis mutant insensitive to ethylene (*ein2*) showed either susceptibility or enhanced resistance to different pathogens (Kunkel and Brooks, 2002). Combinations of azoxystrobin with other fungicides (morpholin or triazole) on winter wheat resulted in better yield (McCabe *et al*., 2001). The treatment with these other fungicide compounds could counteract the effects of reduced ethylene synthesis.

In our experiments, the differences in gene expression patterns between the individual compounds suggest that BTH is a strong trigger of signalling mediated by ethylene and probably SA. Fenpropimorph seems to activate these pathways less strongly but triggers also the JA signalling pathway, whereas azoxystrobin potentially could induce slightly the JA and SA response pathways but inhibit the ethylene pathway. More experiments are needed to confirm these hypotheses as it is well known that cross-talk exists between these signal transduction pathways (Glazebrook, 2001; Kunkel and Brooks, 2002). The study of the early effects of these fungicides (before 24 h) should also help to determine the primary targets, receptors and first steps of the signal transduction pathways involved specifically in wheat depending on the compound applied. The application of strobilurin on *Arabidopsis* mutants in the SAR signal transduction pathways could also help to understand these complex mechanisms more precisely. Strobilurins apparently affect ethylene production by inhibiting induction of the ACC synthase at the post-transcriptional level (Grossmann and Retzlaff, 1997). The specificity of this inhibition might allow a more precise identification of the role of ethylene in this phenomenon.

The induction of defence-related genes by fungicides was surprising and raised some questions about the function of these genes. It is likely that some of them could be not only defence genes against pathogens but contribute to the induction or increase of some metabolic pathways that lead to the resistance of the plant against diverse environmental and biochemical stresses (Wan *et al*., 2002). E.g., they might play a general role in the restoration of the cellular homeostasis.

Gene induction by fungicides and BTH differed dramatically in the field when compared with the greenhouse trial. Plants grown in an agricultural environment are constantly subjected to combinations of stress (drought, wind and pathogen attacks) and our results showed for the three analysed time points expression of a very similar set of defence-related genes as after BTH treatment, except for the *WCI* genes. The impact on gene expression of the three plant protection compounds in the field environment was barely observable and even BTH, which is the strongest enhancer of defence-related genes, did not trigger the transcription of the SAR markers as in the greenhouse trial. The *WCI* genes seemed to be induced by BTH only under greenhouse conditions. This was also confirmed in other field experiments (R. Dudler, personal communication). Probably, stress combinations (pathogen attack or heat shock) in the field had specifically induced defence-related genes. Consequently, the transcriptional machinery might have been altered and the chemically induced genes could not be induced anymore by BTH. This phenomenon could reflect the transcriptional memory of the plant which responds differently according to consecutive stresses, i.e. plants show different expression patterns when submitted to either one type of stress or to a consecutive combination of stressful events (Rizhsky *et al*., 2002; Voelckel and Baldwin, 2004). Therefore, the *WCI* gene expression could have been suppressed by a response to a previous stress event in the field. In a similar manner, the expression of catalase and peroxidase genes is suppressed when drought and heat shock are both applied in tobacco whereas they are over-expressed in the case of a unique stress (Rizhsky *et al*., 2002). It has been shown that treatment with BTH in the field induced resistance of wheat against powdery mildew (Görlach *et al*., 1996). This resistance is possibly triggered by genes not present on our chip or genes expressed at very low level. It could also originate from post-transcriptional changes in gene or protein activity. Strobilurin and morpholin apparently did not induce genes of any defence-related signal transduction pathway in the field.

Our data demonstrate the importance of the environmental growth conditions when testing the effect of agrochemical products on plants, e.g. in studies related to food safety aspects of pesticide

treated crops. It is interesting to note that there are few studies on putative changes of plant metabolism induced by pesticide application. This is in great contrast to the analysis of genetically modified plants where possible changes in plant metabolism are one of the cornerstones in safety assessment.

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